Novel *RHD* alleles with weak hemagglutination and genetic Exon 9 diversity: weak D Types 45.1, 75, and 76

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BACKGROUND: Molecular variant *RHD* allele analysis is best complemented by detailed characterization of the associated D phenotype.
STUDY DESIGN AND METHODS: Variant D types were characterized using molecular typing, *RHD* sequencing, extended serologic D antigen investigations, and flow cytometric D antigen quantification.
RESULTS: We discovered three novel weak D types termed weak D Types 45.1, 75, and 76 with *RHD* nucleotide substitutions coding for amino acid exchanges in predicted intracellular RhD polypeptide stretches; antigen densities of approximately 1.990, 900, and 240 D sites per red blood cell were found, respectively. Adsorption-elution technique–supported D epitope mapping of these three weak D types demonstrated the expression of all tested D epitopes. Initial molecular typing of the three investigated samples by *RHD* gene exon scanning polymerase chain reaction using sequence-specific priming yielded a negative reaction for A1193 located in *RHD* Exon 9 and could be explained by specific mutations for weak D Types 45.1 (C818T, G1195A), 75 (G1194C), and 76 (A1215C).
CONCLUSION: All novel weak D types expressed all tested D epitopes. It is of interest that for weak D Types 45.1, 75, and 76, similar alleles with a maximal divergence of one amino acid only, that is, weak D Types 45, 41, and 68, respectively, have been reported so far.

For decades, Rh blood group antigen D (RH1) typing has been routinely performed by using classical serologic agglutination techniques.[1] However, "weak Ds" are outstanding by their "weak" agglutination behavior, were first called "Du," and were postulated to exhibit quantitative and qualitative differences in comparison to regular D.[1,2] Fifty years after this observation, it is widely accepted that carriers of RhDs with qualitative differences may be immunized by regular D upon transfusion or pregnancy, whereas carriers with only quantitative D variations appear to be immunologically tolerant.[1]

For the latter, Wagner and colleagues[3] reported an original set of 16 different mutations with resulting amino acid substitutions all located in intracellular or transmembraneous protein segments and consequently coined the terminologic entities of "weak D Types 1 to 16." Consequently, the word "weak" with two different meanings in the context of RhD serology needs to be used responsibly:

ABBREVIATION: PCR-SSP = polymerase chain reaction using sequence-specific priming.

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on the one hand, as a description of weak agglutination, which may also be found among most category and partial Ds, and on the other hand, as an integral part of the currently used numerical "weak D type" nomenclature (Rhesus Base, see Web Resources). This numerical terminology has also been influencing the latest version of allele name assignment of blood group genes by the International Society of Blood Transfusion (ISBT, see Web Resources).

Since 1999, an impressive and constantly growing number of new weak D types has been reported. In 2005, half way since their first description until now, weak D Types 31 and 32 were reported, and in 2011 the *RHD* mutation database listed Type 74 as the latest weak D allele recognized so far (Rhesus Base, see Web Resources).[4] The typical discovery of new weak D types starts by the observation of weak hemagglutination and a consecutive rough genetic analysis by polymerase chain reaction using sequence-specific priming (PCR-SSP) method including typing for the most prevalent variant and weak *RHD* alleles, for example, Types 1 to 5, 11, 15, and 17. Finally, *RHD* gene sequencing usually delivers rare and sometimes new *RHD* alleles.[5-7] All new alleles reported here, however, exhibited an additional specific peculiarity, in that they shared a lack of specific amplification dependent on *RHD* specific coding Nucleotide A1193, usually indicative for the presence of *RHD* Exon 9.[6] The new weak D variants presented here are weak D Types 45.1, 75, and 76.

MATERIALS AND METHODS

Investigated blood samples

Sample 1 originated from Labor Wisplinghoff, Cologne, Germany, and Samples 2 and 3 were both patients from the General Hospital and University Clinics in Innsbruck, Austria. All samples showed weak agglutination for RhD using standard serologic techniques. Serologic reinvestigation from a second blood drawing was performed for all samples as described below.

Immunohematology

Blended monoclonal anti-D reagents (DiaClon anti-D, Bio-Rad, Vienna, Austria; Seraclone anti-D blend, Bio-Rad; and anti-D Totem, Diagast, Loos, France) were employed in plate testing and in the anti-human globulin test (IAT) in gel matrix (LISS-antiglobulin test cards, Bio-Rad). Additionally, monoclonal and polyclonal anti-D, anti-C, anti-c, anti-E, and anti-e reagents were used in gel matrix (ABO/Rh for patients; DiaClon Rh-subgroups+K; ABO/Rh; Rh-subgroups+Cw+K, Bio-Rad). Red blood cell (RBC) antibody screening was performed by the IAT in gel matrix (Bio-Rad), as was the direct antiglobulin test (DAT) using monospecific anti-human globulin (anti-IgG, -IgA, -IgM, -C3c, -C3d). For D epitope mapping, 56 human monoclonal anti-D with known D epitope specificity[8] were used in LISS-antiglobulin test cards (Bio-Rad).[4] The following antibody clones were applied: P3x35, P3x61, P3x290, P3x241, P3x249, HM10, HM16, P3x21223B10, and P3x21211F1 (Diagast; provided by K. Göttfert); Lorifa (provided by S. Franz, Ortho Clinical Diagnostics, Vienna, Austria); MS26, NaTH28-3C11, LDM1 (provided by J. Scharrenberg, Bio-Rad); ESD1, LHM76/58, LHM76/59, LHM174/102, LHM50/2B, LHM169/81, LHM77/64, LHM70/45, LHM76/55, LHM59/19, LHM169/80, and LHM57/17 (Bio-Rad); BRAD-2, BRAD-3, BRAD-5, BRAD-7, BRAD-8, FOG-1, 6D10, and 2B6, AB5 (provided by Belinda Kumpel, Bristol Institute for Transfusion Sciences, Bristol, UK); BIRMA D6 and BIRMA D10 (International Blood Group Reference Laboratory/IBGRL, Bristol, UK); H4111B7, BS221, BS226, BS227, BS228, BS229, BS231, and BS232 (provided by M. Ernst, previously Biotest, Dreieich, Germany); BS225 (Sifin, Berlin, Germany); HIMA-37 (provided by G. Halverson, New York Blood Center, New York, NY); MS-201 and LDM3 (Medion, Düdingen, Switzerland); RUM-1 and D175-2 (Immucor, Rödermark, Germany); HIRO-3, HIRO-5, HIRO-9, and HIRO-94 (provided by M. Uchikawa, Tokyo Metropolitan Blood Center, Tokyo, Japan); B9A4-B2A6A6A1A1 (Bio-Rad, Marnes-la-Coquette, France); and NaTH109-1G2 (IQ Products, Groningen, the Netherlands). In case of nonreactivity in IAT, adsorption-elution testing of RBC samples with the respective antibody was performed as previously described.[9]

Flow cytometry

D antigen density of variant D and control RBCs was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as described,[10] using six human IgG anti-D monoclonal antibodies (MoAbs; P3x35, P3x290, HM16, BRAD-3, HIRO-94, and ESD1). The human IgG clone AEVZ5.3 (IBGRL) served as negative control and fluorescein isothiocyanate–labeled Fab fragment goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as secondary reagent. Absolute D antigen density was assessed using ccddee-negative control and CcDdee RBC samples with known D antigen density. For D antigen density calculation, the recommended algorithm was applied.[11]

Molecular biology

Genomic DNA was isolated from ethylenediaminetetraacetate-anticoagulated blood using Nucleon BACC2 reagents (GE Healthcare Europe Austria Branch, Vienna, Austria). First-line *RHD* analysis included routinely performed *RHD* gene exon scanning and weak D genotyping by PCR-SSP methods.[5,6] Both genotyping approaches were performed with commercially available

PCR-SSP genotyping kits (RBC-Ready Gene CDE and D weak, inno-Train, Kronberg i.T., Germany) with enhanced specificity spectrum compared to the original publications, respectively. *RHD* zygosity genotyping was done using commercially available PCR-SSP typing kits (RBC-Ready Gene ZygoFast and D AddOn, inno-Train). Nucleotide sequencing of *RHD* Exons 1 to 10 from genomic DNA of all novel *RHD* variant propositi was performed as described previously.[7]

RESULTS

Routine serology of the investigated RBC samples

Blood samples from one German (Sample 1) and two Austrian individuals (Samples 2 and 3) showed weak agglutination using standard serologic RhD typing. Markedly diminished strength of RBC agglutination was seen with anti-D in plate technique, with only extremely faint positive reactions especially for Sample 3. Using polyclonal and monoclonal anti-D in gel matrix, Samples 1 and 2 showed 3+ to 4+ reactions, whereas Sample 3 displayed 2+ positivity. Further Rh typing yielded a C+c+E–e+ phenotype of Samples 1 and 3 and a C+c–E–e+ result when testing Sample 2. All three samples were C^w–. None of the samples showed unexpected RBC antibodies, and all had a negative DAT.

All three samples typed PCR-SSP negative for *RHD* Nucleotide A1193

Initial molecular typing of the three investigated samples by *RHD* gene exon scanning PCR-SSP yielded a negative reaction for A1193 located in *RHD* Exon 9 and revealed both a hybrid and an upstream Rhesus box indicative of *RHD* heterozygosity in all cases. Sequencing of all 10 *RHD* exons with their flanking intronic sequences of Sample 1 showed two single-nucleotide exchanges in *RHD* Exon 6 and 9 at Nucleotide Positions 818 (Exon 6) and 1195 (Exon 9), whereas Samples 2 and 3 exhibited single-nucleotide exchanges in Exon 9 at Positions 1194 and 1215, respectively (Table 1). All nucleotide exchanges were predicted to cause amino acid substitutions (Table 1), which were expected to be located within either the ninth membrane spanning loop (nucleotide exchange C818T of Exon 6) or the carboxy-terminal end of the RhD protein, located in the intracellular lumen (all Exon 9 nucleotide exchanges), respectively. Based on these findings, weak D type numbers were requested from W.A. Flegel (Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD) and numbers 45.1, 75, and 76 were assigned for Samples 1, 2, and 3, respectively (Table 1). The carrier of weak D Type 75 had a CCDee phenotype and this *RHD* allele may therefore be defined as linked to a *Ce* haplotype. For weak D Type 45.1 and 76, however, no family members were available and phenotypes of the

TABLE 1. Molecular biology of the novel weak D Types 45.1, 75, and 76 in comparison to already known weak D Types 45, 41, and 68

Observed weak D type, novel							Related weak D type, known			RhD protein
Sample	Weak D	Rh phenotype	*RH* haplotype	*RHD* exons	Nucleotide variation	RhD protein	Accession	Weak D	*RHD* exons	Nucleotide variation	
1	Type 45.1	C+c+E–e+	*CDe**	6, 9	C818T, G1195A	A273V, A399T	FR748226	Type 45	9	G1195A	A399T
2	Type 75	C+c–E–e+	*CDe*	9	G1194C	E398D	FR748224	Type 41	9	A1193T	E398V
3	Type 76	C+c+E–e+	*CDe**	9	A1215C	Q405H	FR748225	Type 68	2, 9	T165C, G1213C	Q405E

* Assumed.

TABLE 2. Absolute D antigen density of *RHD* Exon 9 variant and control RBCs

Sample	Allele designation	D antigens per RBC
Weak D Type 45.1	*RHD*(A273V, A399T)	1.990
Weak D Type 75	*RHD*(E398D)	900*
Weak D Type 76	*RHD*(Q405H)	240
Control	Wild-type *RHD*	10.730

* Allele-dependent D antigen reduction probably accentuated by *C* in trans effect in this C+c–E–e+ sample.

carriers were CcDee in both cases. Therefore, the respective *RHD* alleles may only be assumed by statistical chance to be associated with *Ce*.

The quantitative spectrum of D expression of weak D Type 45.1, 75, and 76 RBCs

The absolute D densities of the studied samples confirmed the results obtained by routine serology. As shown in Table 2, weak D Types 45.1 and 75 RBCs showed reduced absolute D antigen densities comparable to those of weak D Types 3 and 1 RBCs, respectively.[4] Of note, the *C* in trans effect may likely have contributed to the low D density of the weak D Type 75 sample (C+c–E–e+), in addition to the allele-specific antigen depression that may in fact be less pronounced when encountered in C+c+ individuals. Weak D Type 76 RBCs had the lowest D density that explained the serologic RhD typing difficulties encountered when using plate technique (see above).

Weak D Types 45.1, 75, and 76 express phenotypes without detectable D epitope loss

The D epitope expression of weak D Types 45.1, 75, and 76 was investigated using a panel of anti-D MoAbs with known epitope specificity in gel matrix IAT. As shown in Table 3, all anti-D MoAbs were reactive in gel matrix IAT with all tested RBC samples except for IgM clone HIMA-37, which required adsorption-elution technique for reactivity with weak D Type 76 RBCs. Unsurprisingly, the observed reaction strength of the anti-D MoAbs paralleled the D density of the respective RBC sample. These results indicated that the novel *RHD* Exon 9 variants expressed quantitatively altered D antigens without apparent D epitope loss.

DISCUSSION

Newly discovered *RHD* alleles may be considered for classification as numerically dubbed weak D types when fulfilling three principal characteristics: 1) Hemagglutination using standard serologic methods is observed to be weaker than the one observed for regular RhD. 2) The observed *RHD* sequence exhibits missense mutations in comparison to the *RHD* wild-type sequence encoding amino acid substitutions in transmembraneous or intracellular parts of the RhD polypeptide. 3) Carriers of such alleles do not develop alloanti-D when confronted with regular RhD.[3] This rough definition and numerical weak D classification system, however, includes reasonable impact for controversial discussion, because exceptions of the above-described three rules were observed since the original description of the molecular basis of weak D phenotypes.[3] The novel *RHD* alleles described here all fulfilled above-mentioned inclusion criteria 1) to 3) and were dubbed weak D Types 45.1, 75, and 76, accordingly. This numerical terminology offers a comprehensive overview over a distinct group of *RHD* alleles, and facilitates the comparison of different weak D types.

With respect to comparisons, it is of interest that for weak D Types 45.1, 75, and 76, similar alleles (Table 1) with a maximal divergence of one amino acid, for example, weak D Types 45, 41, and 68, respectively, have been reported so far (Rhesus Base, see Web Resources). However, with respect to antigen density, no data for weak D Types 45, 41, and 68 were available. Consequently the direct effects on RhD protein expression depending on single amino acid exchanges, for weak D Types 75 and 76 even at identical positions compared to weak D Types 41 and 68, may not be analyzed.

Antigen densities for weak D Types 45.1, 75, and 76 were determined to be 1.990, 900, and 240 D sites per RBC. Hence, the newly described samples exhibited D densities clearly distinct from upper limits of approximately 5.900 to 7.000 for weak D Type 20 (G.F. Körmöczi, unpublished; Rhesus Base, see Web Resources) and lower limits of approximately 30 for weak D Types 26 and *RHD*(del Ex10, 5.4 kb), respectively.[7,12] Therefore, with respect to D antigen density, weak D Types 45.1 and 75 may be apostrophized as "average." D expression of weak D Type 76 RBCs was about half of the one seen in weak D Type 2 cells. Such samples may easily be mistaken for D– when serologic typing is performed only by direct agglutination in plate technique.[4] In any case, all weak D types should be considered to be capable for the induction of anti-D alloimmunization when transfused to D– recipients.[7,13]

All three novel weak D Types 45.1, 75, and 76 alleles share a remarkable common feature, in that all of them typed PCR-SSP negative for coding nucleotide A1193 located in Exon 9 of the *RHD* gene. Sequence analysis exhibited mutations at 1193 nearby coding Nucleotides 1195, 1194, and 1215, which offer an excellent explanation for the lack of specific PCR-SSP amplification in the observed alleles (Table 1). Most certainly, lack of A1193-specific amplification was due to the reverse orientation of the Nucleotide A1193–specific amplification primer and its recognition sequence covering coding Nucleotides A1193 to T1220, respectively. Apparently, even single-

TABLE 3. D epitope mapping of *RHD* Exon 9 variant and R_1r control RBCs

			Reaction in the antihuman globulin test in gel matrix*			
Monoclonal anti-D	Immunoglobulin class	D epitope	R_1r	Weak D Type 45.1	Weak D Type 75	Weak D Type 76
LHM169/81	IgG	1.1	4	4	3	2
BRAD-7	IgG	1.1	4	3	3	2
LHM70/45	IgG	1.2	3	2	2	2
LHM174/102	IgG	1.2	4	4	3	2
P3x249	IgG	2.1	4	4	3	3
BS227	IgG	2.2	4	4	3	2
BRAD-8	IgG	2.2	4	4	4	2
H41 11B7	IgG	3.1	4	4	3	3
P3x290	IgG	3.1	4	4	4	3
LHM76/55	IgG	3.1	4	4	3	2
ESD1	IgG	4.1	4	4	3	3
NaTH109-1G2	IgG	5.2	4	4	4	3
BS229	IgG	5.4	4	4	4	2
BS231	IgG	5.4	4	4	4	3
P3x35	IgG	5.4	4	3	3	2
P3x241	IgG	5.4	4	4	4	3
MS-201	IgM	6.1	4	4	2	2
RUM-1	IgM	6.1	4	4	3	2
P3x61	IgM	6.1	4	4	2	2
D175-2	IgM	6.1	4	4	3	2
BRAD-3	IgG	6.2	4	4	4	3
Lorifa	IgG	6.2	3	2	2	2
6D10	IgG	6.2	4	4	4	3
BS221	IgG	6.3	4	4	4	2
BS228	IgG	6.3	4	4	4	2
LHM169/80	IgG	6.3	4	4	3	3
AB5	IgG	6.3	4	4	4	3
HIRO-9	IgG	6.3	3	3	3	2
LHM50/2B	IgG	6.3	4	4	3	3
LHM57/17	IgG	6.3	3	2	2	2
HM16	IgG	6.4	4	4	4	3
BS225	IgM	6.4	4	4	2	1
BS226	IgM	6.4	4	4	2	2
NaTH28-3C11	IgM	6.4	4	3	2	1
BS232	IgM	6.4	4	4	2	2
B9A4-B2A6A6A1A1	IgM	6.4	4	4	2	1
HIRO-5	IgG	6.5	4	4	3	2
LDM1	IgM	6.5	4	3	2	0.5
LDM3	IgM	6.5	4	2	2	0.5
HM10	IgM	6.6	4	4	2	1
FOG-1	IgG	6.7	3	2	2	1
HIRO-94	IgG	6.7	4	4	4	3
2B6	IgG	6.8	4	3	3	2
BRAD-5	IgG	6.8	4	4	3	3
LHM59/19	IgG	8.1	4	3	3	3
LHM76/58	IgG	8.1	4	3	3	3
P3x212 11F1	IgM	8.2	4	3	2	0.5
BRAD-2	IgG	9.1	4	4	3	2
MS26	IgG	9.1	4	4	4	3
P3x212 23B10	IgM	9.1	4	2	0.5	0.5
LHM77/64	IgG	9.1	4	4	3	3
BIRMA D6	IgG	9.1	4	4	3	3
LHM76/59	IgG	15.1	4	4	3	3
HIRO-3	IgG	16.1	3	3	3	2
BIRMA D10	IgM	NA	4	4	3	2
HIMA-37	IgM	NA	4	0.5	0.5	0†

* Numbers denote reaction strength: weakly to strongly positive (0.5-4), negative (0).
† Weakly positive upon adsorption-elution testing.
NA = not available.

nucleotide exchanges of the investigated DNA abolished regular primer binding with a consequent lack of amplification, which may be interpreted as indicative for the high specificity achieved by PCR-SSP technology in general.[6]

WEB RESOURCES

1. Rhesus Base; http://www.uni-ulm.de/~fwagner/RH/RB2/

2. International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology; ISBT on a global scale [website]; http://www.isbtweb.org > Working Parties > Red Cell Immunogenetics and Blood Group Terminology.

CONFLICT OF INTEREST

SS is employed at inno-Train GmbH, Kronberg i.T., Germany. All other authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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